A

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Docket No: 29473/35635

PATENT APPLICATION TRANSMITTAL UNDER 37 C.F.R. 1.53

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Transmitted herewith for filing is the patent application of

Inventors: Hans REICHENBACH, Gerhard HOEFLE, Klaus GERTH and Heinrich STEINMETZ

Title: EPOTHILONES C, D, E AND F, PREPARATION AND COMPOSITIONS

1. Type of Application

☒ This is a new application for a

☒ utility patent.

☐ design patent.

2. Application Papers Enclosed

1	Title Page
17	Pages of Specification (excluding Claims, Abstract, Drawings & Sequence Listing)
5	Pages of Claims
1	Pages of Abstract
4	Sheets of Drawings (Figs. 1 to 5)
☐	Formal
☒	Informal

CERTIFICATION UNDER 37 CFR 1.10

I hereby certify that this Patent Application Transmittal and the documents referred to as enclosed therewith are being deposited with the United States Postal Service on **May 17, 1999**, in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231 utilizing the "Express Mail Post Office to Addressee" service of the United States Postal Service under Mailing Label No. EM099538212US.

Richard Zimmermann

3. **Declaration or Oath**

- ☐ Enclosed
  - ☐ Executed by (check all applicable boxes)
    - ☐ Inventor(s)
    - ☐ Legal representative of inventor(s) (37 CFR 1.42 or 1.43)
    - ☐ Joint inventor or person showing a proprietary interest on behalf of inventor who refused to sign or cannot be reached
      - ☐ The petition required by 37 CFR 1.47 and the statement required by 37 CFR 1.47 are enclosed. See Item 5D below for fee.

- ☒ Not enclosed - the undersigned attorney or agent is authorized to file this application on behalf of the applicant(s). An executed declaration will follow.

4. **Additional Papers Enclosed**

- ☒ Preliminary Amendment
- ☐ Information Disclosure Statement
- ☒ Declaration of Biological Deposit
- ☐ Computer readable copy of sequence listing containing nucleotide and/or amino acid sequence
- ☐ Microfiche computer program
- ☐ Verified statement(s) claiming small entity status under 37 CFR 1.9 and 1.27
- ☐ Associate Power of Attorney
- ☐ Verified translation of a non-English patent application
- ☐ An assignment of the invention
- ☒ Return receipt postcard
- ☐ Other

5. **Priority Applications Under 35 USC 119**

Certified copies of applications from which priority under 35 USC 119 is claimed are listed below and

☐ are attached.

☒ will follow.

COUNTRY	APPLICATION NO.	FILED
Germany	196 47 580.5	18 November 1996
Germany	197 07 506.1	26 February 1997

6. **Filing Fee Calculation (37 CFR 1.16)**

A. ☒ **Utility Application**

CLAIMS AS FILED - INCLUDING PRELIMINARY AMENDMENT (IF ANY)						
			SMALL ENTITY		OTHER THAN A SMALL ENTITY	
	NO. FILED	NO. EXTRA	RATE	FEE	RATE	FEE
BASIC FEE				$380.00		$760.00
TOTAL	16 -20	= 0	X 9 =		X 18 =	
INDEP.	10 - 3	= 7	X 39 =		X 78 =	546.00
☐ First Presentation of Multiple Dependent Claim			+ 130 =		+ 260 =	
Filing Fee:					**OR**	1,306.00

B. ☐ **Design Application ($155.00/$310.00)** Filing Fee: $ _____

C. ☐ **Plant Application ($240.00/$480.00)** Filing Fee: $ _____

D. **Other Fees**

☐ Recording Assignment [Fee -- **$40.00** per assignment] $ _____

☐ Petition fee for filing by other than all the inventors or person on behalf of the inventor where inventor refused to sign or cannot be reached [Fee -- **$130.00**] $ _____

☐ Other $ _____

Total Fees Enclosed **$1,306.00**

7. **Method of Payment of Fees**

☒ Enclosed check in the amount of: $1,306.00

☐ Charge Deposit Account No. 13-2855 in the amount of: $_____
A copy of this Transmittal is enclosed.

☐ Not enclosed

8. **Deposit Account and Refund Authorization**

The Commissioner is hereby authorized to charge any deficiency in the amount enclosed or any additional fees which may be required during the pendency of this application under 37 CFR 1.16 or 37 CFR 1.17 or under other applicable rules (except payment of issue fees), to Deposit Account No. 13-2855. A copy of this Transmittal is enclosed.

Please refund any overpayment to Marshall, O'Toole, Gerstein, Murray & Borun at the address below.

Please direct all future communications to James P. Zeller, at the address below.

Respectfully submitted,

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By: _____
James P. Zeller
Reg. No: 28,491

May 17, 1999

PATENT

IN THE UNITED STATES PATENT
AND TRADEMARK OFFICE

Applicant: Reichenbach et al. Serial No.: To be assigned Filed: Herewith (May 17, 1999) For: EPOTHILONES C, D, E AND F, PREPARATION AND COMPOSITIONS Continuation of International Application No.: PCT/EP97/06442 filed November 18, 1997 Group Art Unit: To be assigned Examiner: To be assigned	"EXPRESS MAIL" mailing label No. EM099538212US. Date of Deposit: May 17, 1999 I hereby certify that this paper (or fee) is being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 CFR §1.10 on the date indicated above and is addressed to: Assistant Commissioner for Patents, Box Patent Application, Washington, D.C. 20231 Richard Zimmermann

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Box Patent Application
Washington, DC 20231

Sir:

Please amend this application as follows.

IN THE SPECIFICATION:

Page 1, immediately following the title, please insert the following:

--This is a continuation of International Application No. PCT/EP97/06442 filed November 18, 1997, the entire disclosure of which is incorporated be reference.--

Page 1, line 30 please delete "me".

Page 1, line 33 please delete "epothione" and substitute --epothilone-- therefor.

Page 2, line 4 please delete "and".

Page 7, line 31 please substitute --30°C-- for "30C".

IN THE CLAIMS:

Claim 8, line 1 please delete "6" and substitute --7-- therefor.

Claim 9, line 1 please delete "6 or".

Claim 13, line 1 please delete "11" and substitute --12-- therefor.

Claim 14, line 1 please delete "11 or".

Please amend claims 15 and 16 as follows:

15. (Amended) Composition for plant protection in agriculture and forestry and/or in horticulture [, consisting of] comprising at least one [or more of the compounds] compound according to [one of the preceding claims or one or more of these compounds] claim 1, optionally in addition to at least one [or more customary carrier(s)] carrier and/or [diluents(s)] diluent.

16. (Amended) Therapeutic composition[, in particular for use as a cytostatic, consisting of] comprising at least one [or more of the compounds] compound according to [one or more of the preceding claims or one or more of the compounds according to one

or more of the preceding claims] claim 1, optionally in addition to at least one [or more customary carrier(s)] carrier and/or [diluent(s)] diluent.

REMARKS

By the foregoing amendments to the specification, a cross-reference to the parent international application has been provided, and obvious typographical errors have been corrected at page 1, lines 30 and 33, at page 2, line 4, and at page 7, line 31. Further, the claims have been amended to correct obvious errors in the dependencies of claims 8, 9, 13, and 14, to eliminate multiple dependencies (claims 9, 14, 15, and 16), and to conform claims 15 and 16 to U.S. practice.

The specification as filed herewith is identical to the international application as filed but for the omission from page 1 and the abstract of identifying headings; the substitution of the word --and-- for "with" at page 3, line 29; the omission of the duplicate phrase "Epothilone E R = H" from page 5, line 10; the substitution of --2 m^3/hr-- for "0.1 NL/m^3" at page 7, line 7; the substitution of --MHz-- for "MH_Z" at page 15, line 7; and the substitution of --1 l/min-- for "0.1 vvm" at page 16, line 11.

The claims as filed herewith (before the present amendments thereto) are identical to those filed during international preliminary examination. The filing fee

for the present application has been calculated on the basis of the claims as amended herein.

Respectfully submitted,

MARSHALL, O'TOOLE, GERSTEIN,
MURRAY & BORUN

May 17, 1999 By ______________________
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JOINT INVENTORS

"EXPRESS MAIL" mailing label No. EM099538212US.
Date of Deposit: May 17, 1999
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Richard Zimmermann

APPLICATION FOR UNITED STATES LETTERS PATENT

SPECIFICATION

TO ALL WHOM IT MAY CONCERN:

Be it known that we, Hans REICHENBACH, a citizen of Germany, residing at Mascheroder Weg 1, D-38124 Braunschweig, Germany; Gerhard HOEFLE, a citizen of Germany, residing at Mascheroder Weg 1, D-38124 Braunschweig, Germany; Klaus GERTH, a citizen of Germany, residing at Mascheroder Weg 1, D-38124 Braunschweig, Germany; and Heinrich STEINMETZ, a citizen of Germany, residing at Mascheroder Weg 1, D-38124 Braunschweig, Germany have invented a new and useful EPOTHILONES C, D, E AND F, PREPARATION AND COMPOSITIONS, of which the following is a specification.

Epothilones C, D, E and F, Preparation and Compositions

The present invention relates to epothilones C, D, E and F, their preparation and their use for the production of therapeutic compositions and compositions for plant protection.

Epothilones C and D

According to one embodiment, the invention relates to epothilones [C and D] which are obtainable in that

(a) Sorangium cellulosum DSM 6773 is cultured in a manner known per se in the presence of an adsorber resin,

(b) the adsorber resin is removed from the culture and washed with a water/methanol mixture,

(c) the washed adsorber resin is eluted with methanol and the eluate is concentrated to give a crude extract,

(d) the concentrate obtained is extracted with ethyl acetate, the extract is concentrated and partitioned between methanol and hexane,

(e) the methanolic phase is concentrated to give a raffinate and the concentrate is fractionated on a Sephadex column,

(f) a fraction containing metabolic products of the microorganism employed is obtained,

(g) the fraction obtained is chromatographed on a C18 reverse phase to me a methanol/water mixture and, sequentially

- after a first fraction containing epothilone A and
- a second fraction containing epothione B
- a third fraction containing a first further epothilone

and

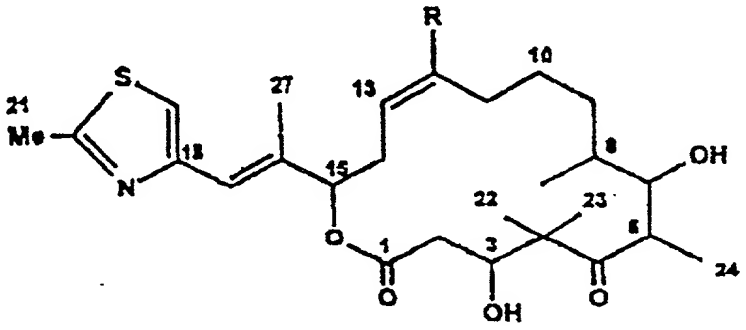
- a fourth fraction containing a second further epothilone are obtained and

(h1) and the epothilone of the first further fraction and/or

(h2) the epothilone of the second further fraction are isolated.

The invention further relates to an epothilone [C] of the empirical formula $C_{26}H_{39}NO_5S$, characterized by the ^1H- and ^{13}C-NMR spectrum as in Table 1.

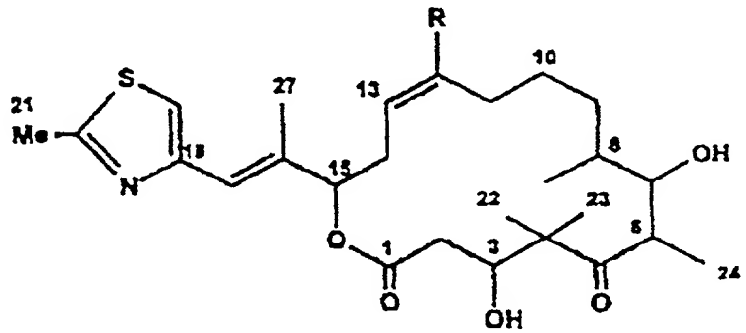
The invention furthermore relates to epothilone C of the formula:



Epothilone C R = H

The invention furthermore relates to epothilone [D] of the empirical formula $C_{27}H_{41}NO_5S$, characterized by the ^1H- and ^{13}C-NMR spectrum as in Table 1.

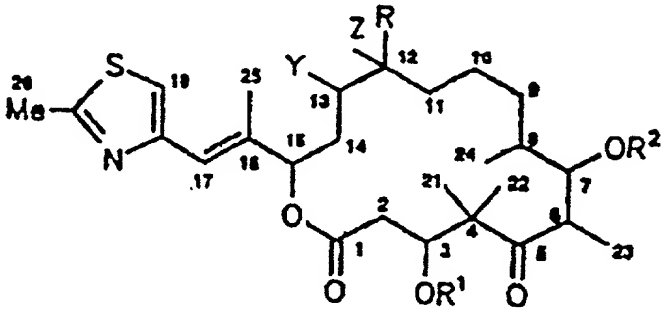
The invention furthermore relates to epothilone D of the formula:



Epothilone D R = CH_3

Epothilones C and D can be used for the preparation of the compounds of the following formula 1, where

for their derivatization reference can be made to the derivatization methods described in WO-A-97/19 086.



In the above formula 1:

R = H, C_{1-4}-alkyl;

R^1, R^2, R^3, R^4, R^5 = H, C_{1-6}-alkyl,
C_{1-6}-acylbenzoyl,
C_{1-4}-trialkylsilyl,
benzyl,
phenyl,
C_{1-6}-alkoxy-,
C_6-alkyl-, hydroxy- and halogen-substituted benzyl or phenyl;

where two of the radicals R^1 to R^5 can also combine to form the group $-(CH_2)_n-$ with n = 1 to 6 and the alkyl or acyl groups contained in the radicals are straight-chain or branched radicals;

Y and Z are either identical or different and are each hydrogen, halogen, such as F, Cl, Br or I, pseudohalogen, such as -NCO, -NCS or $-N_3$, OH, O-(C_{1-6})-acyl, O-(C_{1-6})-alkyl, O-benzoyl. Y and Z can also be the O atom of an epoxide, epothilone A and B not being claimed, or form one of the C-C bonds of a C=C double bond.

Thus the 12,13-double bond can be selectively

- hydrogenated, for example catalytically or with diimine, a compound of the formula 1 being obtained with Y = Z = H; or
- epoxidized, for example with dimethyldioxirane or a peracid, a compound of the formula 1 being obtained with Y and Z = -O-; or
- converted into the dihalides, dipseudohalides or diazides, a compound of the formula 1 being obtained with

Y and Z = Hal, pseudo-hal or N_3.

Epothilones E and F

According to a further embodiment the invention relates to a biotransformant of epothilone A, which is obtainable in that

(a) *Sorangium cellulosum* DSM 6773 is cultured in a manner known per se in the presence of an adsorber resin, removed from the adsorber resin and, if appropriate, the total amount or a part of the separated culture is treated with a methanolic solution of epothilone A,

(b) the culture treated with epothilone A is incubated and then treated with adsorber resin,

(c) the adsorber resin is separated from the culture, eluted with methanol and the eluate is concentrated to give a crude extract,

(d) the crude extract is partitioned between ethyl acetate and water, the ethyl acetate phase is separated off and concentrated to give an oil,

(e) the oil is chromatographed on a reverse phase under the following conditions:

column material:	Nucleosil 100 C-18 7 µm
column dimensions:	250 × 16 mm
eluent:	methanol/water = 60 : 40
flow rate:	10 ml/min

and fractions having a content of biotransformant and which can be detected by UV extinction at 254 nm and have an R_t value of 20 min are separated off and the biotransformants are isolated.

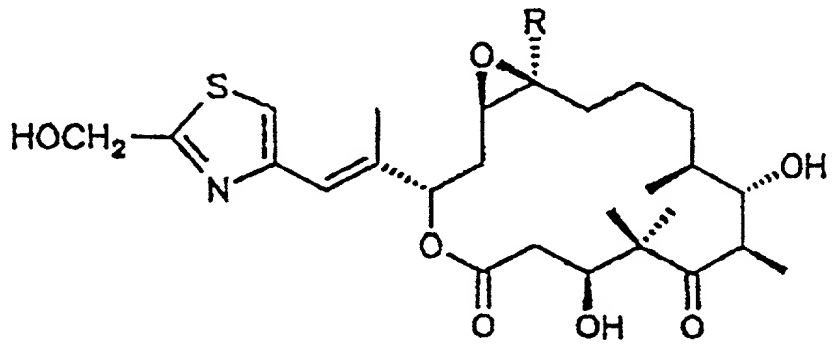
The invention furthermore relates to a biotransformant of epothilone A of this type, which is obtainable in that in stage (a) a culture is separated off which is three or four or more days old.

The invention furthermore relates to a biotransformant of epothilone A of this type, which is obtainable in that in stage (b) incubation is carried out for one or two or more days.

The invention furthermore relates to a compound of the empirical formula $C_{26}H_{39}NO_7S$, characterized by the

following ^{1}H-NMR spectrum (300 MHz, $CDCl_3$): delta = 2.38 ($2\text{-}H_a$), 2.51 ($2\text{-}H_b$), 4.17 (3-H), 3.19 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, $9\text{-}H_2$, $10\text{-}H_2$, $11\text{-}H_2$), 2.89 (12-H), 3.00 (13-H), 1.88 ($14\text{-}H_a$), 2.07 ($14\text{-}H_b$), 5.40 (15-H), 6.57 (17-H), 7.08 (19-H), 4.85 ($21\text{-}H_2$), 1.05 ($22\text{-}H_3$), 1.32 ($23\text{-}H_3$), 1.17 ($24\text{-}H_3$), 0.97 ($25\text{-}H_3$), 2.04 ($27\text{-}H_3$)

The invention furthermore relates to a compound (epothilone E) of the formula:



Epothilone E R = H

According to a further embodiment, the invention relates to a biotransformant of epothilone B, which is obtainable in that

(a) *Sorangium cellulosum* DSM 6773 is cultured in a manner known per se in the presence of an adsorber resin, separated from the adsorber resin and, if appropriate, the total amount or a part of the separated culture is treated with a methanolic solution of epothilone B,

(b) the culture treated with epothilone B is incubated and then treated with adsorber resin,

(c) the adsorber resin is separated from the culture, eluted with methanol and the eluate is concentrated to give a crude extract,

(d) the crude extract is partitioned between ethyl acetate and water, the ethyl acetate phase is separated off and concentrated to give an oil,

(e) the oil is chromatographed on a reverse phase under the following conditions:

column material: Nucleosil 100 C-18 7 µm

column dimensions:	250 × 16 mm
eluent:	methanol/water = 60 : 40
flow rate:	10 ml/min

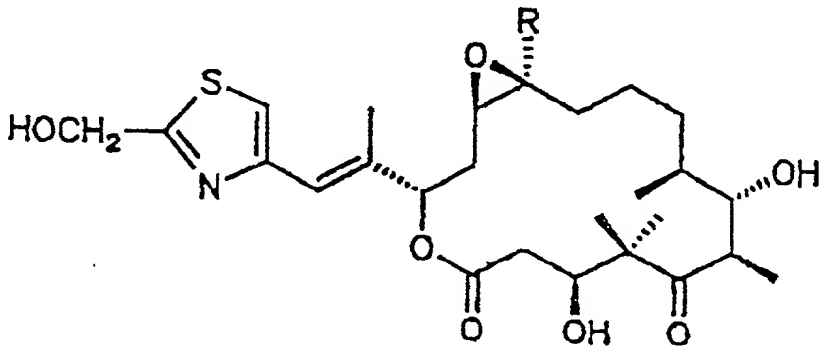
and fractions having a content of biotransformant and which can be detected by UV extinction at 254 nm and have an R_t value of 24.5 min are separated off and the biotransformants are isolated.

The invention furthermore relates to a biotransformant of epothilone B of this type, which is obtainable in that in stage (a) a culture is separated off which is three or four or more days old.

The invention furthermore relates to a biotransformant of epothilone B of this type, which is obtainable in that in stage (b) incubation is carried out for one or two or more days.

The invention furthermore relates to a compound of the empirical formula $C_{27}H_{41}NO_7S$, characterized by the following ^{1}H-NMR spectrum (300 MHz, $CDCl_3$): delta = 2.37 (2-H_a), 2.52 (2-H_b), 4.20 (3-H), 3.27 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.78 (13-H), 1.91 (14-H), 2.06 (14-H_b), 5.42 (15-H), 6.58 (17-H), 7.10 (19-H), 4.89 (21-H_2), 1.05 (22-H_3), 1.26 (23-H_3), 1.14 (24-H_3), 0.98 (25-H_3), 1.35 (26-H_3), 2.06 (27-H_3).

The invention furthermore relates to a compound (epothilone F) of the formula:



Epothilone F R = CH_3

Preparation and compositions

The compounds or epothilones according to the invention are obtainable by the abovementioned measures.

The invention furthermore relates to compositions for plant protection in agriculture, forestry and/or horticulture, consisting of one or more of the abovementioned epothilones C, D, E and F or consisting of one or more of the abovementioned epothilones in addition to one or more customary carrier(s) and/or diluent(s).

The invention finally relates to therapeutic compositions, consisting of one or more of the abovementioned compounds or one or more of the abovementioned compounds in addition to one or more customary carrier(s) and/or diluent(s). In particular, these compositions can show cytotoxic activities and/or bring about immunosuppression and/or be employed for the control of malignant tumours, it being particularly preferably possible for them to be used as cytostatics.

In the following, the invention is illustrated and described in greater detail by the description of some selected working examples.

Examples

Example 1:
Epothilones C and D

A. Production strain and culture conditions according to the epothilone basic patent DE-B-41 38 042.

B. Production with DSM 6773

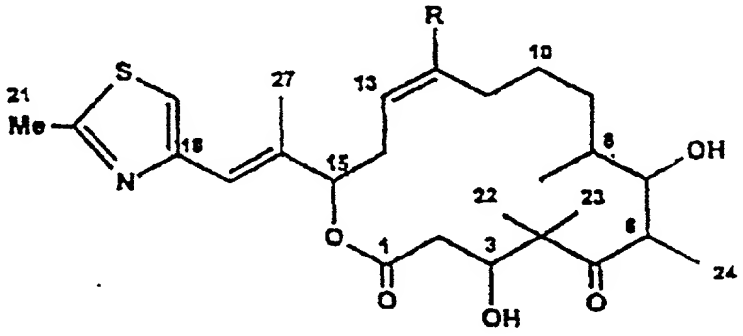
75 l of culture are grown as described in the basic patent and used for the inoculation of a production fermenter with 700 l of production medium consisting of 0.8% starch, 0.2% glucose, 0.2% soya flour, 0.2% yeast extract, 0.1% $CaCl_2 \times 2H_2O$, 0.1% $MgSO_4 \times 7H_2O$, 8 mg/l of Fe-EDTA, pH = 7.4 and optionally 15 l of Amberlite XAD-16 adsorber resin. The fermentation lasts 7 - 10 days at 30C , aeration with $2\ m^3/hr$. By controlling the speed of rotation, the pO_2 is kept at 30%.

C. Isolation

The adsorber resin is separated from the culture using a 0.7 m^2, 100 mesh process filter and freed from polar concomitants by washing with 3 bed volumes of

water/methanol 2:1. By elution with 4 bed volumes of methanol, a crude extract is obtained which is evaporated in vacuo until the water phase appears. This is extracted three times with the same volume of ethyl acetate. Evaporation of the organic phase affords 240 g of crude extract, which is partitioned between methanol and heptane in order to separate off lipophilic concomitants. By evaporation in vacuo, 180 g of raffinate are obtained from the methanol phase and are fractionated into three portions on Sephadex LH-20 (column 20 x 100 cm, 20 ml/min of methanol). The epothilones are contained in the fraction of a total of 72 g eluted with a 240 - 300 min retention time. To separate the epothilones, the fraction is chromatographed in three portions on Lichrosorb RP-18 (15 µm, column 10 x 40 cm, eluent 180 ml/min methanol/water 65:35). After epothilone A and B, epothilone C, with R_t = 90-95 min, and epothilone D, 100-110 min, are eluted and after evaporation in vacuo obtained in a yield of 0.3 g each as colourless oils.

D. Physical properties



Epothilone C R = H

Epothilone D R = CH_3

Epothilone C

$C_{26}H_{39}NO_5S$ [477]

ESI-MS: (positive ions): 478.5 for $[M+H]^+$

1H and 13C see NMR table

TLC: R_f = 0.82

TLC aluminium foil 60 F 254 Merck, eluent: dichloromethane/methanol = 9:1

Detection: UV extinction at 254 nm. Spraying with vanillin-sulphuric acid reagent, blue-grey coloration on heating to 120°C.

HPLC: R_t = 11.5 min

Column: Nucleosil 100 C-18 7 µm, 125 × 4 mm

Eluent: methanol/water = 65:35

Flow rate: 1 ml/min

Detection: diode array

Epothilone D

$C_{27}H_{41}NO_5S$ [491]

ESI-MS: (positive ions): 492.5 for $[M+H]^+$

1H and 13C see NMR table

TLC: R_f = 0.82

TLC aluminium foil 60 F 254 Merck, eluent: dichloromethane/methanol = 9:1

Detection: UV extinction at 254 nm. Spraying with vanillin-sulphuric acid reagent, blue-grey coloration on heating to 120°C.

HPLC: R_t = 15.3 min

Column: Nucleosil 100 C-18 7 µm, 125 × 4 mm

Eluent: methanol/water = 65:35

Flow rate: 1 ml/min

Detection: diode array

Table 1: ^{1}H- and ^{13}C-NMR data of epothilone C and epothilone D in [D_6] DMSO at 300 MHz

	Epothilone C			Epothilone D		
H atom	δ (ppm)	C atom	δ (ppm)	δ (ppm)	C atom	δ (ppm)
		1	170.3		1	170.1
2-Ha	2.38	2	38.4	2.35	2	39.0
2-Hb	2.50	3	71.2	2.38	3	70.8
3-H	3.97	4	53.1	4.10	4	53.2
3-OH	5.12	5	217.1	5.08	5	217.4
6-H	3.07	6	45.4	3.11	6	44.4
7-H	3.49	7	75.9	3.48	7	75.5
7-OH	4.46	8	35.4	4.46	8	36.3
8-H	1.34	9	27.6	1.29	9	29.9
9-Ha	1.15	10	30.0	1.14	10	25.9
9-Hb	1.40	11	27.6	1.38	11	31.8*
10-Ha	1.15*	12	124.6	1.14*	12	138.3
10-Hb	1.35*	13	133.1	1.35*	13	120.3
11-Ha	1.90	14	31.1	1.75	14	31.6*
11-Hb	2.18	15	76.3	2.10	15	76.6
12-H	5.38**	16	137.3		16	137.2
13-H	5.44**	17	119.1	5.08	17	119.2
14-Ha	2.35	18	152.1	2.30	18	152.1
14-Hb	2.70	19	117.7	2.65	19	117.7
15-H	5.27	20	164.2	5.29	20	164.3
17-H	6.50	21	18.8	6.51	21	18.9
19-H	7.35	22	20.8	7.35	22	19.7
21-H_3	2.65	23	22.6	2.65	23	22.5
22-H_3	0.94	24	16.7	0.90	24	16.4
23-H_3	1.21	25	18.4	1.19	25	18.4
24-H_3	1.06	27	14.2	1.07	26	22.9
25-H_3	0.90			0.91	27	14.1
26-H_3				1.63		
27-H_3	2.10			2.11		

*, ** assignment interchangeable

Example 2:

Epothilone A and 12,13-bisepi-epothilone A from epothilone C

50 mg of epothilone A are dissolved in 1.5 ml of acetone and treated with 1.5 ml of a 0.07 molar solution of dimethyldioxirane in acetone. After standing at room temperature for 6 hours, the mixture is evaporated in vacuo and the residue is separated by preparative HPLC on silica gel (eluent: methyl tert-butyl ether/petroleum ether/methanol 33:66:1).

Yield:

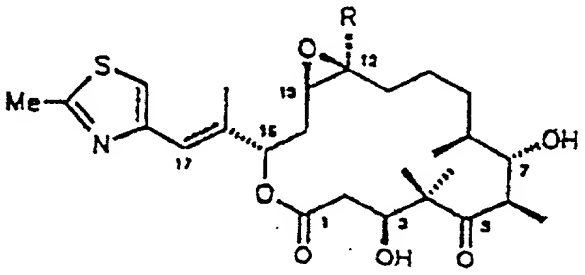
25 mg of epothilone A, R_t = 3.5 min (analyt. HPLC, 7 µm, column 4 × 250 mm, eluent see above, flow rate 1.5 ml/min)

and

20 mg of 12,13-bisepi-epothilone A, R_t = 3.7 min, ESI-MS (pos. ions)

m/e = 494 $[M+H]^+$

^{1}H-NMR in [D_4] methanol, selected signals: delta = 4.32 (3-H), 3.79 (7-H), 3.06 (12-H), 3.16 (13-H), 5.54 (15-H), 6.69 (17-H), 1.20 (22-H), 1.45 (23-H).



12,13-bisepi-epothilone A R = H

Example 3:

Epothilone E and F, novel biotransformation products of epothilones A and B.

Production strain:

The production strain *Sorangium cellulosum* So

ce90 was isolated in July 1985 in GBF from a soil sample from the banks of the Zambesi and deposited on 28.10.91 in the German Collection for Microorganisms under No. DSM 6773.

The characterization of the producer and the culture conditions are described in:
Höfle, G.; N. Bedorf, K. Gerth & H. Reichenbach: Epothilones, processes for their preparation and compositions containing them. DE 41 38 042 A1, laid open on 27th May 1993.

Formation of epothilones E and F during fermentation:

A typical fermentation proceeds in the following manner: A 100 l bioreactor is filled with 60 l of medium (0.8% starch; 0.2% glucose; 0.2% soya flour; 0.2% yeast extract; 0.1% $CaCl_2 \times 2H_2O$; 0.1% $MgSO_4 \times 7H_2O$; 8 mg/l of Fe-EDTA; pH 7.4). 2% of adsorber resin (XAD-16, Rohm & Haas) are additionally added. The medium is sterilized by autoclaving (2 hours, 120°C). Inoculation is carried out with 10 l of a preculture grown in the same medium (additionally 50 mM HEPES buffer pH 7.4) in a shaker flask (160 rpm, 30°C). Fermentation is carried out at 32°C with a stirrer speed of 500 rpm and an introduction of 0.2 Nl per m^3 per hour of air, the pH is kept at 7.4 by addition of KOH. The fermentation lasts 7 to 10 days. The epothilones formed are continuously bound to the adsorber resin during the fermentation. After separating off the culture broth (e.g. by screening in a process filter), the resin is washed with 3 bed volumes of water and eluted with 4 bed volumes of methanol. The eluate is concentrated to dryness and taken up in 700 ml of methanol.

HPLC analysis of the XAD eluate:

In relation to the starting volume of the reactor (70 l), the eluate is concentrated 100:1. The analysis is carried out using a 1090 HPLC unit from Hewlett Packard. To separate the constituents, a microbore column (125/2 Nucleosil 120-5 C_{18}) from Machery-Nagel (Düren) is used. Elution is carried out using a gradient of water/aceto-

nitrile from initially 75:25 up to 50:50 after 5.5 minutes. This ratio is maintained up to the 7th minute, in order to then increase it up to the 10th minute to 100% acetonitrile.

Measurement is carried out at a wavelength of 250 nm and a bandwidth of 4 nm. The diode array spectra are measured in the wavelength range from 200 to 400 nm. In the XAD eluate, two novel substances with R_t 5.29 and R_t 5.91 stand out, whose adsorption spectra are identical with those of epothilones A and B (Fig. 1; E corresponds to A, F corresponds to B). These substances are only formed in traces under the given fermentation conditions.

Biotransformation of epothilone A and B to epothilone E and F:

A 500 ml culture of So ce90, 4 days old and maintained with adsorber resin, is used for the specific biotransformation. 250 ml of this are transferred to a sterile 1 l Erlenmeyer flask leaving behind the XAD. A methanolic solution of a mixture of a total of 36 mg of epothilone A + 14 mg of B is then added and the flask is incubated on a shaking rack for two days at 30°C and 200 rpm. The formation of the epothilones E and F is analysed directly from 10 µl of the centrifuged culture supernatant (Fig. 2). The conversion takes place only in the presence of the cells and is dependent on the cell densities employed and the time. Kinetics of the conversion are shown for epothilone A in Fig. 3.

Isolation of epothilone E and F

To isolate epothilone E and F, three shaker flask batches from the biotransformation (see above) are combined and shaken with 20 ml of XAD-16 for 1 h. The XAD is obtained by screening and eluted with 200 ml of methanol. The eluate is evaporated in vacuo to give 1.7 g of crude extract. This is partitioned between 30 ml of ethyl acetate and 100 ml of water. On evaporation in vacuo, 330 mg of an oily residue are obtained from the ethyl acetate phase, which are chromatographed in five runs on a 250 × 20 mm RP-18 column (eluent: methanol/water 58:42, detection 254 nm).

Yield: epothilone E 50 mg
F 10 mg

Biological action of epothilone E:

In cell cultures, the concentration was determined which reduces the growth by 50% (IC_{50}) and compared with the values for epothilone A.

Cell line	IC_{50} (ng/ml)	
	Epothilone E	Epothilone A
HeLa.KB-3.1 (human)	5	1
Mouse fibroblasts, L929	20	4

Epothilone E

$C_{26}H_{39}HO_7S$ [509]

ESI-MS: (positive ions): 510.3 for $[M+H]^+$

TLC: R_f = 0.58

TLC aluminium foil 60 F 254 Merck, eluent: dichloromethane/methanol = 9:1

Detection: UV extinction at 254 nm. Spraying with vanillin-sulphuric acid reagent, blue-grey colouration on heating to 120°C.

HPLC: R_t = 5.0 min

Column: Nucleosil 100 C-18 7 µm, 250 × 4 mm

Eluent: methanol/water = 60:40

Flow rate: 1.2 ml/min

Detection: diode array

^{1}H-NMR (300 MHz, $CDCl_3$): delta = 2.38 (2-H_a), 2.51 (2-H_b), 4.17 (3-H), 3.19 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.89 (12-H), 3.00 (13-H), 1.88 (14-H_a), 2.07 (14-H_b), 5.40 (15-H), 6.57 (17-H), 7.08 (19-H), 4.85 (21-H_2), 1.05 (22-H_3), 1.32 (23-H_3), 1.17 (24-H_3), 0.97 (25-H_3), 2.04 (27-H_3)

Epothilone F

$C_{27}H_{41}NO_7S$ [523]

ESI-MS: (positive ions): 524.5 for $[M+H]^+$

TLC: R_f = 0.58

TLC aluminium foil 60 F 254 Merck, eluent: dichloromethane/methanol = 9:1

Detection: UV extinction at 254 nm. Spraying with vanillin-sulphuric acid reagent, blue-grey

colouration on heating to 120°C.

HPLC: R_t = 5.4 min

Column: Nucleosil 100 C-18 7 µm, 250 × 4 mm

Eluent: methanol/water = 60:40

Flow rate: 1.2 ml/min

Detection: diode array

^{1}H-NMR (300 MHz , $CDCl_3$): delta = 2.37 (2-H_a), 2.52 (2-H_b), 4.20 (3-H), 3.27 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.78 (13-H), 1.91 (14-H), 2.06 (14-H_b), 5.42 (15-H), 6.58 (17-H), 7.10 (19-H), 4.89 (21-H_2), 1.05 (22-H_3), 1.26 (23-H_3), 1.14 (24-H_3), 0.98 (25-H_3), 1.35 (26-H_3), 2.06 (27-H_3).

Example 4:

Preparation of epothilone E and F by biotransformation with *Sorangium cellulosum* So ce90

1) Carrying out the biotransformation:

For the biotransformation, a culture of *Sorangium cellulosum* So ce90 is used which has been shaken for four days in the presence of 2% XAD 16 adsorber resin (Rohm and Haas, Frankfurt/M.) at 30°C and 160 rpm. The culture medium has the following composition in g/litre of distilled water: potato starch (Maizena), 8; glucose (Maizena), 8; defatted soya flour, 2; yeast extract (Marcor), 2; ethylenediaminetetraacetic acid, iron(III) sodium salt, 0.008; $MgSO_4 \times 7H_2O$, 1; $CaCl_2 \times 2\ H_2O$, 1; HEPES 11.5. The pH is adjusted to 7.4 before autoclaving with KOH. The XAD is separated from the culture by sieving through a stainless steel sieve (200 µm mesh width). The bacteria are sedimented by centrifugation for 10 min at 10,000 rpm and the pellet is resuspended in 1/5 of the culture supernatant. Epothilone A or epothilone B in methanolic solution is then added to the concentrated bacterial suspension in a concentration of 0.5 g/ litre. The culture is cultured further as described above. To analyse the biotransformation, a 1 ml sample is taken at the desired times, 0.1 ml of XAD is added and the sample is shaken at 30°C for 30 min. The XAD is eluted with methanol. The eluate is concentrated to dryness and taken up again in 0.2 ml of methanol. This sample is analysed

by means of HPLC.

Fig. 4) Kinetics of the biotransformation of epothilone A to epothilone E

Fig. 5) Kinetics of the biotransformation of epothilone B to epothilone F.

2) Preparation of epothilone E by biotransformation of 1 g of epothilone A.

The strain *Sorangium cellulosum* So ce90 is grown for four days in 8.5 l of the above medium (but without XAD addition) in a 10 litre bioreactor at 30°C, a speed of rotation of 150 rpm and an introduction of 1 l/min of air.

The culture is then concentrated to 3 l by crossflow filtration. For this purpose, 0.6 m^2 of a membrane having a pore size of 0.3 µm are used.

The concentrated culture is transferred to a 4 litre bioreactor and a methanolic solution of 1 g of epothilone A in 10 ml of methanol is added. The culture is then cultured further for a period of time of 21.5 h. The temperature is 32°C, the stirrer speed is 455 rpm and the introduction of air takes place at 6 l/min. At the time of harvesting, 100 ml of XAD is added and the mixture is incubated further for 1 h. The XAD is separated from the cells by screening and exhaustively eluted with methanol. The concentrated eluate is analysed by means of HPLC.

Balancing of the biotransformation:

Epothilone A employed:	1000 mg	= 100%
Epothilone A recovered after 21.5 h:	53.7 mg	= 5.4%
Epothilone E formed after 21.5 h:	661.4 mg	= 66.1%
Epothilone A completely decomposed:		= 28.5%

Experiment 5:

The epothilones according to the invention were tested with cell cultures (Table 2) and for promotion of polymerization (Table 3).

Table 2:

Epothilone tests with cell cultures

Epothilone	A 493	B 507	C 477	D 491	E 509	F 523
		IC-50	[ng/ml]			
Mouse fibroblasts L 929	4	1	100	20	20	1.5
human tumor cell lines:						
HL-60 (leukaemia)	0.2	0.2	10	3	1	0.3
K-562 (leukaemia)	0.3	0.3	20	10	2	0.5
U-937 (lymphoma)	0.2	0.2	10	3	1	0.2
KB-3.1 (carcinoma of the cervix)	1	0.6	20	12	5	0.5
KB-V1 (carcinoma of the cervix multires	0.3	0.3	15	3	5	0.6
A-498 (carcinoma of the kidney)	-	1.5	150	20	20	3
A-549 (carcinoma of the lung)	0.7	0.1	30	10	3	0.1

Table 3:

Polymerization test with epothilones

Parameter: Time up to the half-maximal polymerization of the control

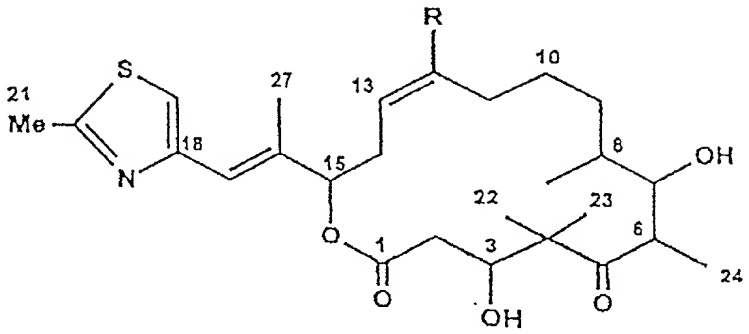
Measurement:	w	x	y	z	Agent [s]	Agent [%]
Control	200	170	180	210	190	100
Epothilone A	95	60	70	70	74	39
Epothilone B		23	25	30	26	14
Epothilone C	125	76	95	80	94	49
Epothilone D	125	73	120		106	56
Epothilone E	80	60	50	45	59	31
Epothilone F	80	40	30	50	50	26

Standard test with 0.9 mg of tubulin/ml and 1 µM sample concentration

The polymerization test is an in vitro test using purified tubulin from pigs' brain. Evaluation is carried out photometrically. Polymerization-promoting substances such as the epothilones reduce the time up to which half-maximal polymerization has taken place, i.e. the shorter the time, the more active the compound. w, x, y and z are four independent experiments, the relative activity is expressed in the last column in % of the control; again the lowest values indicate the best activity. The ranking list corresponds reasonably accurately to that found in cell cultures.

Patent Claims

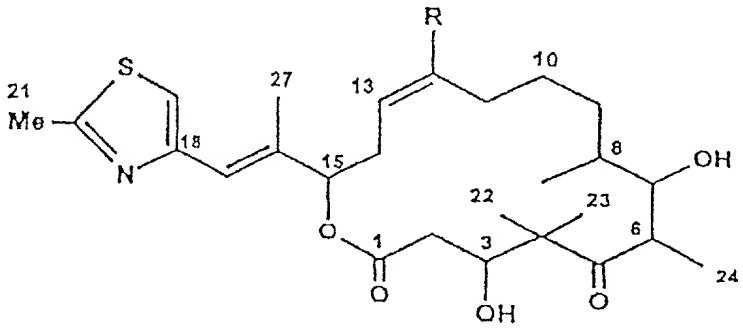
1. Epothilone C of the formula:



Epothilone C R = H

2. Epothilone of the empirical formula $C_{26}H_{39}NO_5S$, characterized by the 1H- and 13C-NMR spectrum as in Table 1.

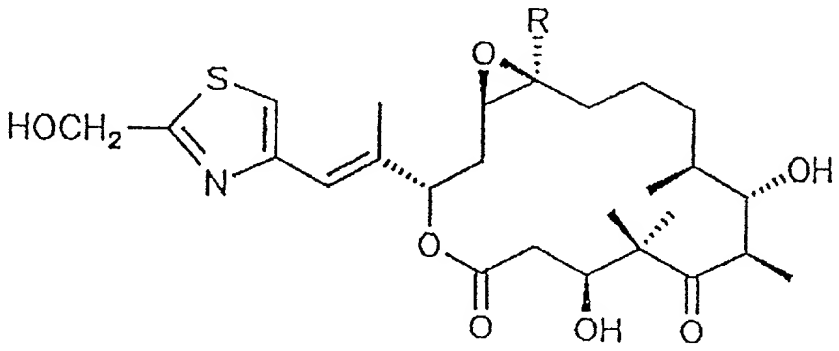
3. Epothilone D of the formula:



Epothilone D R = CH_3

4. Epothilone of the empirical formula $C_{27}H_{41}NO_5S$, characterized by the ^1H- and ^{13}C-NMR spectrum as in Table 1.

5. Compound (epothilone E) of the formula:



Epothilone E R = H

6. Compound of the empirical formula $C_{26}H_{39}NO_7S$, characterized by the following ^1H-NMR spectrum (300 MHz, $CDCl_3$): delta = 2.38 (2-H_a), 2.51 (2-H_b), 4.17 (3-H), 3.19 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.89 (12-H), 3.00 (13-H), 1.88 (14-H_a), 2.07 (14-H_b), 5.40 (15-H), 6.57 (17-H), 7.08 (19-H), 4.85 (21-H_2), 1.05 (22-H_3), 1.32 (23-H_3), 1.17 (24-H_3), 0.97 (25-H_3), 2.04 (27-H_3).

7. Biotransformant of epothilone A, obtainable in that
   (a) Sorangium cellulosum DSM 6773 is cultured in a manner known per se in the presence of an adsorber resin, removed from the adsorber resin and, if appropriate, the total amount or a part of the separated culture is treated with a methanolic solution of epothilone A,
   (b) the culture treated with epothilone A is incubated and then treated with adsorber resin,

(c) the adsorber resin is separated from the culture, eluted with methanol and the eluate is concentrated to give a crude extract,

(d) the crude extract is partitioned between ethyl acetate and water, the ethyl acetate phase is separated off and concentrated to give an oil,

(e) the oil is chromatographed on a reverse phase under the following conditions:

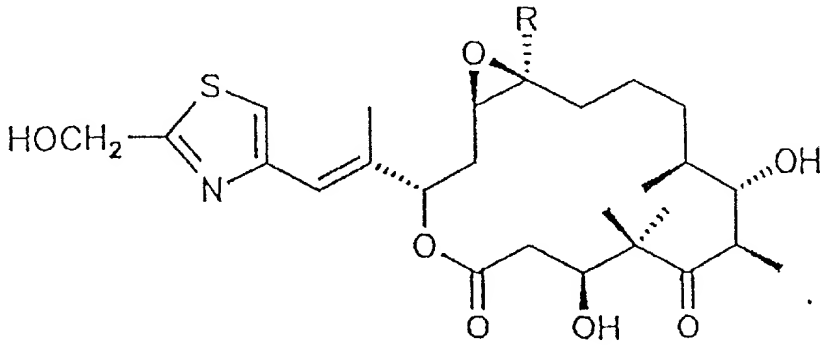
column material:	Nucleosil 100 C-18 7 µm
column dimensions:	250 x 4 mm
eluent:	methanol/water = 60 : 40
flow rate:	1.2 ml/min

and fractions having a content of biotransformant and which can be detected by UV extinction at 254 nm and have an R_t value of 5.0 min are separated off and the biotransformants are isolated.

8. Biotransformant of epothilone A according to Claim 6, characterized in that in stage (a) a culture is separated off which is three or four or more days old.

9. Biotransformant of epothilone A according to Claim 6 or 7, obtainable in that in stage (b) incubation is carried out for one or two or more days.

10. Compound (epothilone F) of the formula:



Epothilone F R = CH_3

11. Compound of the empirical formula $C_{27}H_{41}NO_7S$, characterized by the following ^1H-NMR spectrum (300 MHz, $CDCl_3$): delta = 2.37 (2-H_a), 2.52 (2-H_b), 4.20 (3-H), 3.27 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.78 (13-H), 1.91 (14-H), 2.06 (14-H_b), 5.42 (15-H), 6.58 (17-H), 7.10 (19-H), 4.89 (21-H_2), 1.05 (22-H_3), 1.26 (23-H_3), 1.14 (24-H_3), 0.98 (25-H_3), 1.35 (26-H_3), 2.06 (27-H_3).

12. Biotransformant of epothilone B, obtainable in that
    (a) Sorangium cellulosum DSM 6773 is cultured in a manner known per se in the presence of an adsorber resin, separated from the adsorber resin and, if appropriate, the total amount or a part of the separated culture is treated with a methanolic solution of epothilone B,
    (b) the culture treated with epothilone B is incubated and then treated with adsorber resin,
    (c) the adsorber resin is separated from the culture, eluted with methanol and the eluate is concentrated to give a crude extract,
    (d) the crude extract is partitioned between ethyl acetate and water, the ethyl acetate phase is separated off and concentrated to give an oil,
    (e) the oil is chromatographed on a reverse phase under the following conditions:

column material:	Nucleosil 100 C-18 7 µm
column dimensions:	250 x 4 mm
eluent:	methanol/water = 60 : 40
flow rate:	1.2 ml/min

    and fractions having a content of biotransformant and which can be detected by UV extinction at 254 nm and have an R_t

value of 5.4 min are separated off and the biotransformants are isolated.

13. Biotransformant according to Claim 11, obtainable in that in stage (a) a culture is separated off which is three or four more days old.

14. Biotransformant according to Claim 11 or 12, obtainable in that in stage (b) incubation is carried out for one or two more days.

15. Composition for plant protection in agriculture and forestry and/or in horticulture, consisting of one or more of the compounds according to one of the preceding claims or one or more of these compounds in addition to one or more customary carrier(s) and/or diluents(s).

16. Therapeutic composition, in particular for use as a cytostatic, consisting of one or more of the compounds according to one or more of the preceding claims or one or more of the compounds according to one or more of the preceding claims in addition to one or more customary carrier(s) and/or diluent(s).

Abstract

Epothilones C, D, E and F, Preparation and Compositions

The present invention relates to epothilones C, D, E and F, their preparation and their use for the production of therapeutic compositions and compositions for plant protection.

Fig. 1 HPLC analysis of an XAD eluate at the end of a fermentation.

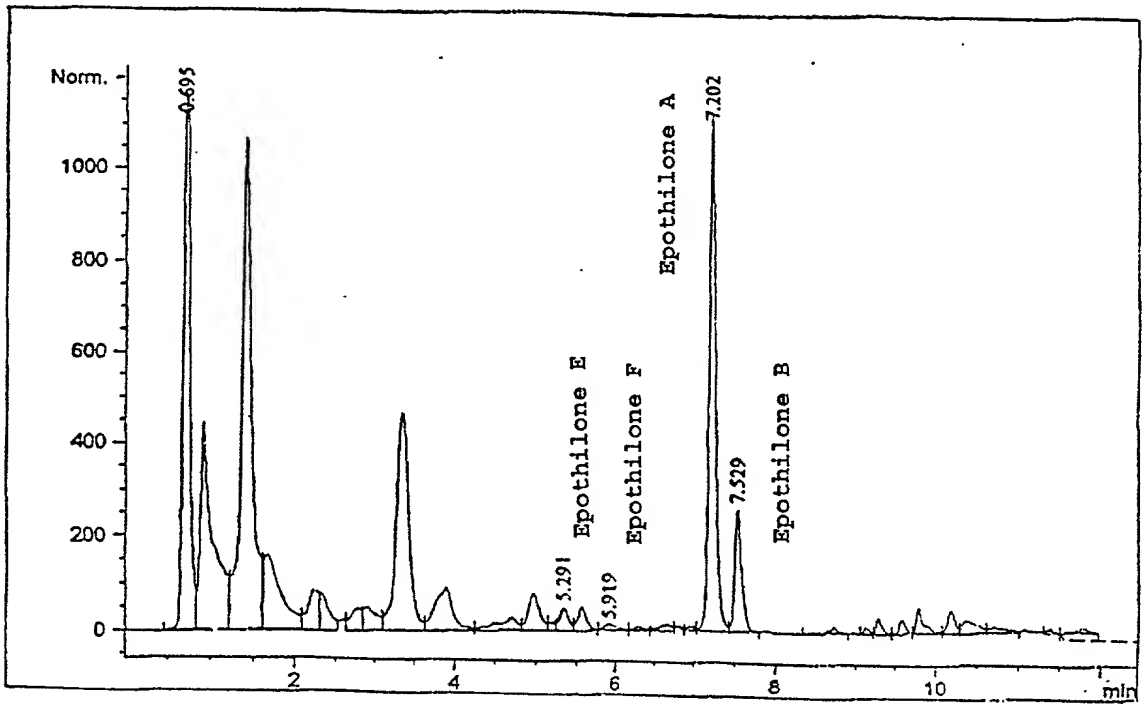


Fig. 2 Enrichment of epothilone E and F in a fermentation broth after feeding of a mixture of epothilone A and B, analysed after incubation for 48 hours.

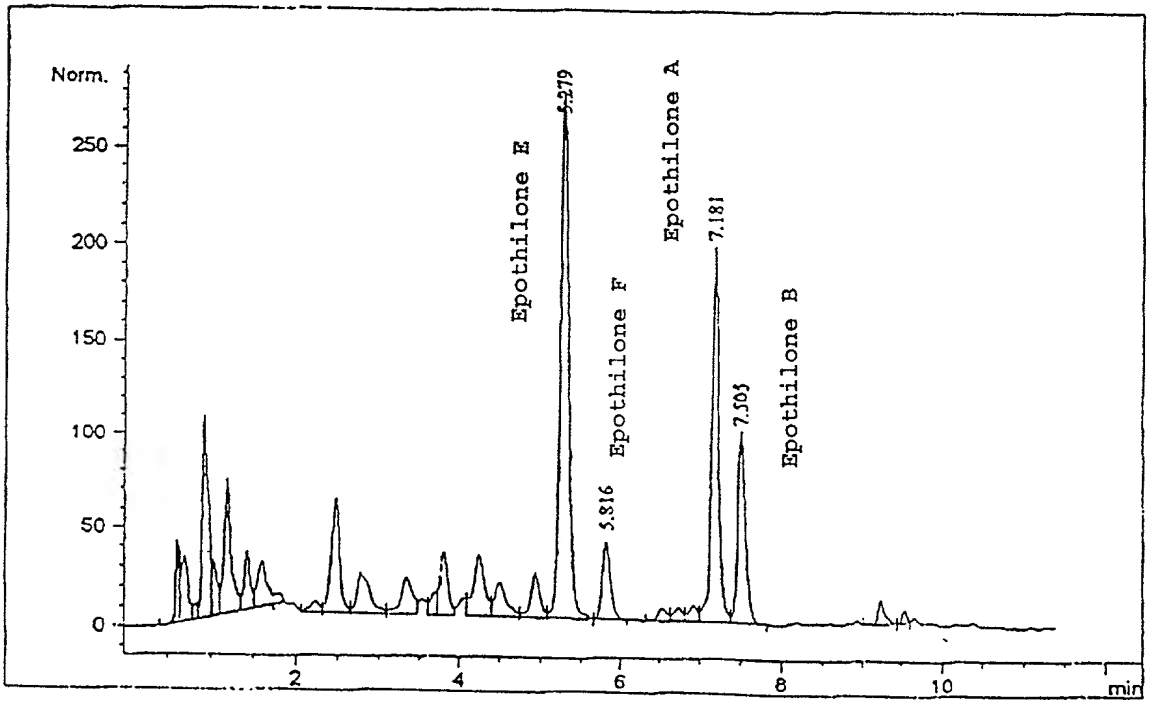


Fig. 3 Kinetics of the biotransformation of epothilone A to epothilone E by *Sorangium cellulosum* So ce90.

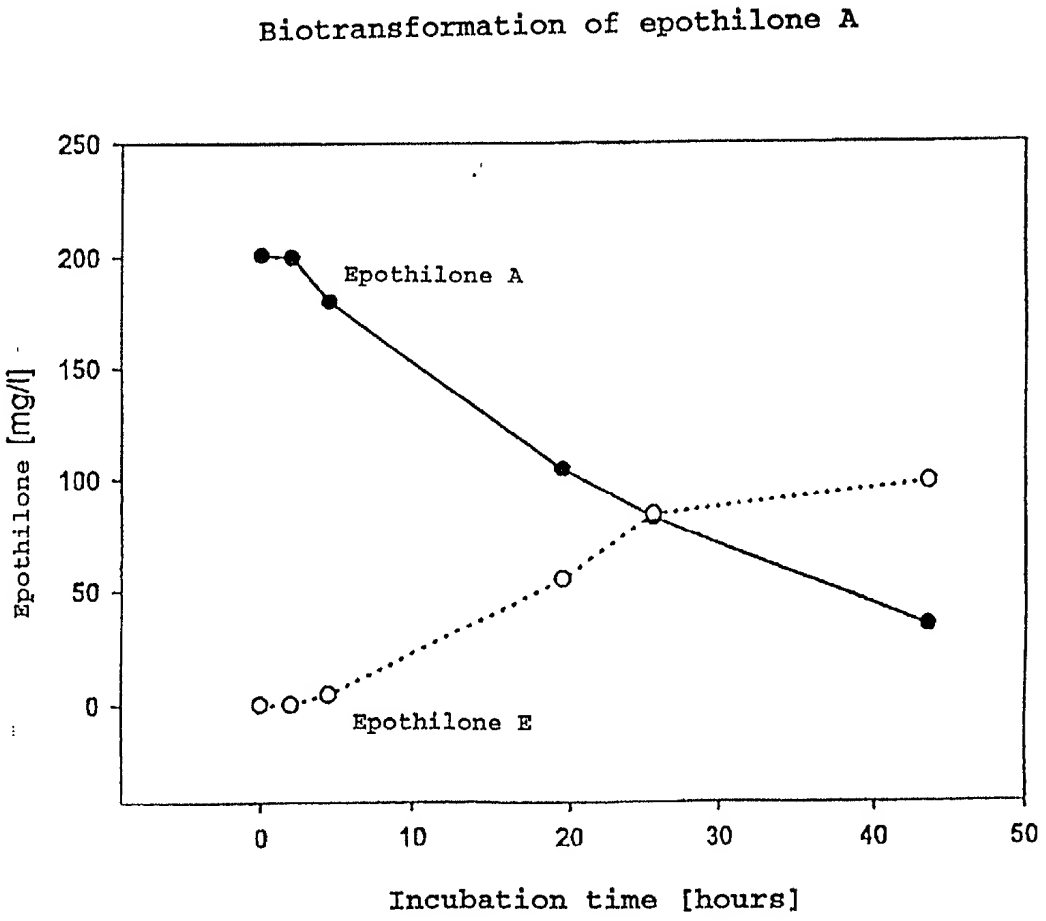
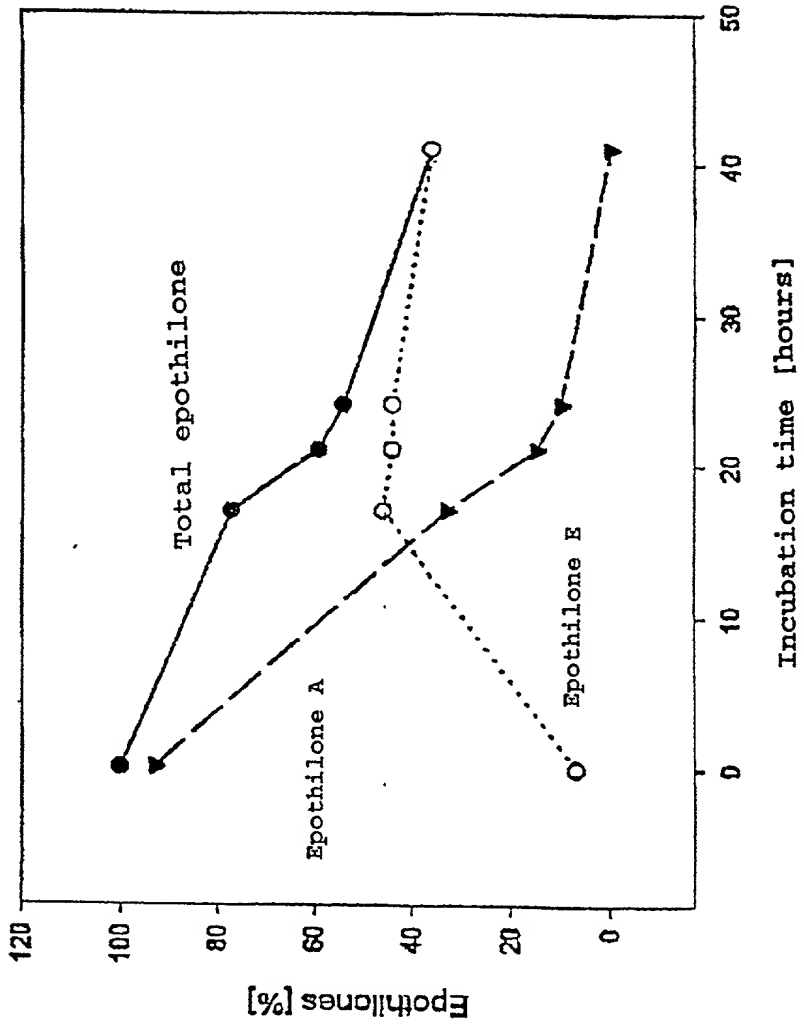


Fig. 4 Biotransformation of epothilone A to epothilone E



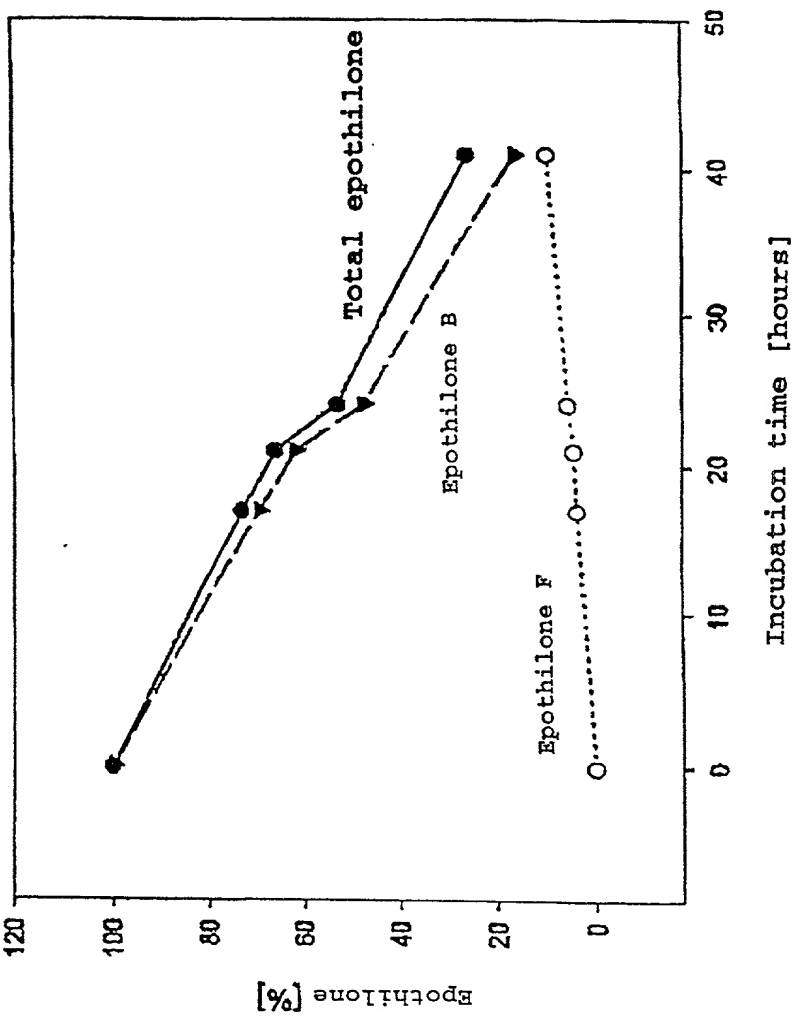


Fig. 5 Biotransformation of epothilone B to epothilone E